Development of a combined RT-PCR: NGS assay for microRNA biomarkers

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IzoSeq

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Overview

The ability of miRNAs to act as diagnostic biomarkers could be expanded by availability of improved methodologies to detect and analyse these molecules. We have therefore developed an assay with the ability to selectively analyse pools of miRNAs, using the specificity of PCR to select targets and the power of NGS to reveal isomiRs of the chosen targets in a total assay time of two days.

Introduction and Methodology

- MicroRNAs (miRNAs) are small RNAs, ~22 nucleotides in length
- Regulate gene expression by targeting partially complementary mRNA transcripts for degeneration or inhibition of translation
- Processed from stem-loop precursors
- Differential cleavage and non-template additions create many variants for each miRNA, known as **isomiRs** (**Figure 1**)

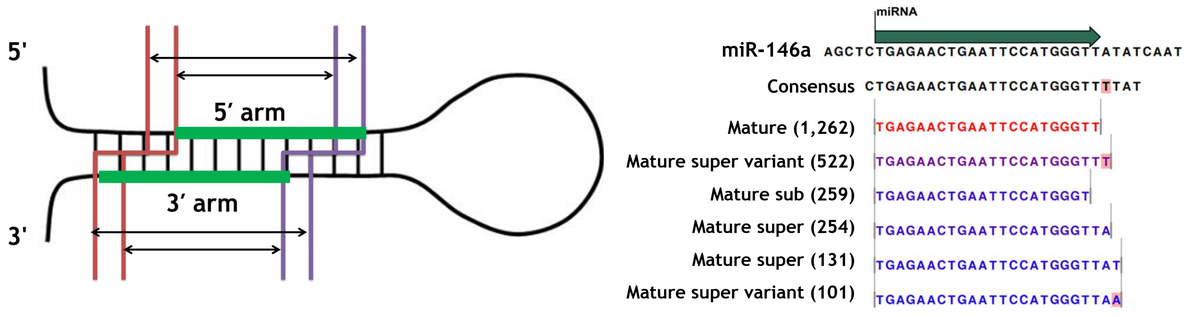
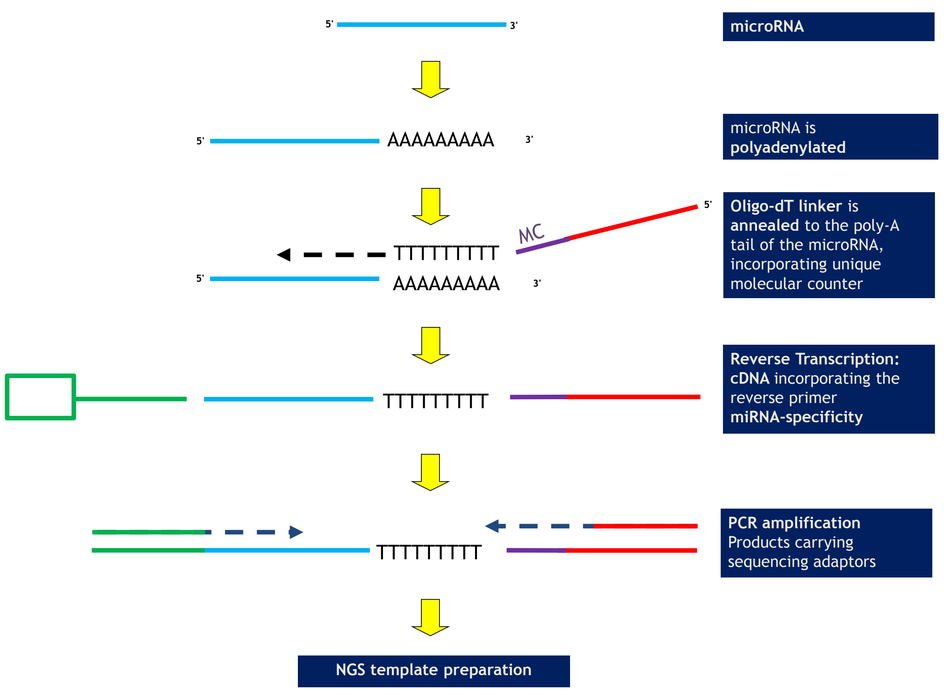


Figure 1. miRNA isomiRs. (**Left**) The position at which the RNase enzymes responsible for miRNA processing, Drosha (brown) and Dicer (purple), cleave the pre-miRNA sites, can vary slightly. This generates variants or isomiRs. (**Right**) Examples of isomiR variants for miR146a.

- miRNAs are stable in blood: Biomarker potential
- IsomiRs increase information content and provide tissue specificity
- The standard detection methods are RNA-Seq and RT-PCR
- RNA-Seq gives global RNA analysis but an overabundance of data that swamps the specific information required.
- RT-PCR focuses on the miRNA of interest but lacks isomiR data
- IzoSeq combines the specificity of RT-PCR with NGS to allow isomiR detection for miRNAs of interest (**Figure 2**).



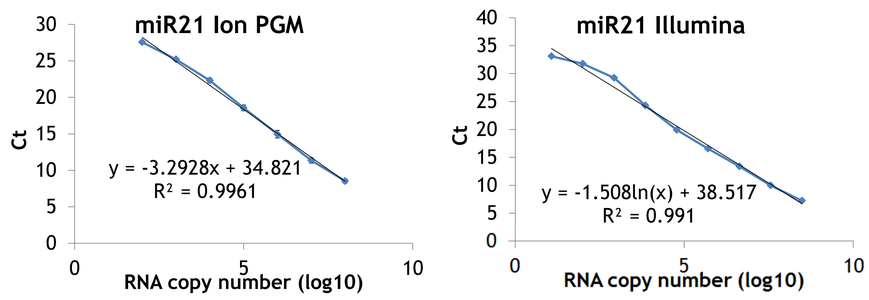
TECHNOLOGY	Sensitivity	Specificity	Quantitative	Throughput	Cost per assay	Cost per miRNA
Microarray	Low	Low	Medium	High	Medium-high	Low
RT-qPCR	High	High	High	Medium	Low-medium	Medium
NGS	High	Very high	Medium	Very high	High	Very low
IzoSeq	Very high	Very high	Absolute	Medium	Medium	Medium

Figure 2. Libraries are constructed for analysis on the Ion Torrent or Illumina platform. (**Top**) Total cellular RNA is polyadenylated and reverse transcribed using an oligo-d(T) primer containing a counter sequence. The cDNA for the miRNA of interest is selected and PCR amplified. The resulting product incorporates the required sequencing adapters. (**Bottom**) This technique is much more cost effective in comparison to existing techniques.

Results and Discussion

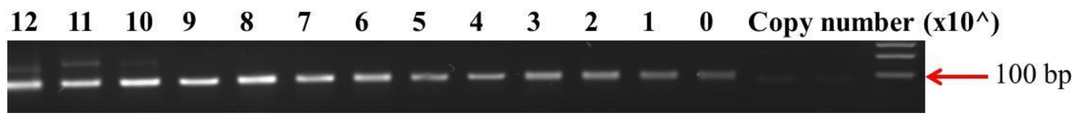
qPCR cycling conditions and limit of detection

- qPCR analysis of miR-21 showed PCR efficiency of 101% and r^2 of 0.9961 across a 7-log dilution series.
- Compatible with Ion Torrent and Illumina sequencing platforms.



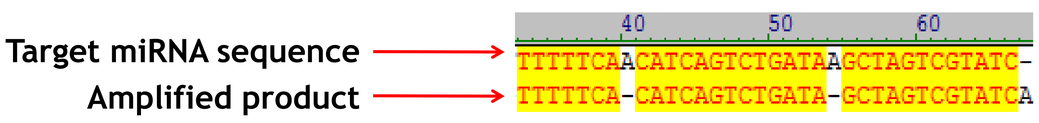
End-point PCR cycling conditions for library construction

- Assay end-point PCR protocols were optimised for library production.



Sanger sequencing of libraries

- Conventional sequencing confirmed specific amplification of target miRNAs from a pool of total cellular RNA.



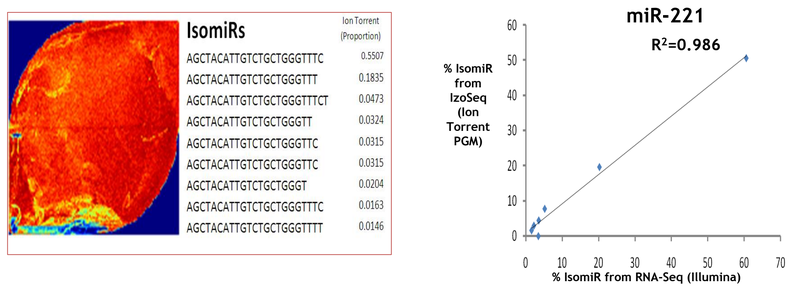
NGS analysis using the Ion PGM

- Detection of miR-21isomiRs expressed in cells derived from circulating endothelial progenitors

Ion PGM sequence	Reads
UAGCUUAUCAGACUGAUGUUG	6064
UAGCUUAUCAGACUGAUGUG	3011
UAGCUUAUCAGACUGAUGUAG	2647
UAGCUUAUCAGACUGUGUAG	2399
CAGAAGCUUAUCAGACUGAUGUUG	1379
UAGCUUAUCAGACUGAUGUUGA	1025
UAGCUUAUCAGACUGAUGUGA	638
UAGCUUAUCAGCUGUGUAG	574
UAGCUUAUCAGACUGAUGUUGAC	479
UAGCUUAUCAGACUGAUGUAGA	449

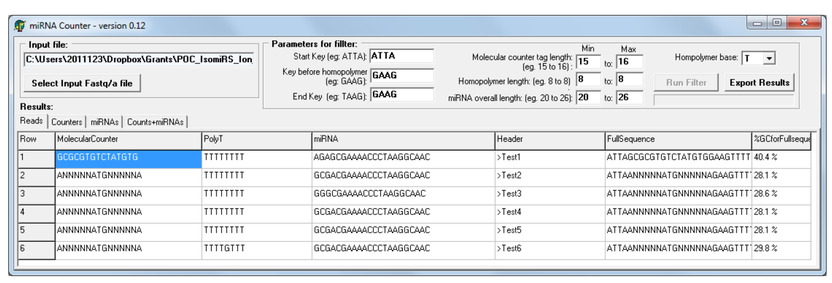
miRBase sequence	Reads
UAGCUUAUCAGACUGAUGUUGAC	641
UAGCUUAUCAGACUGAUGUUGA	577
UAGCUUAUCAGACUGAUGUUGACUG	124
UAGCUUAUCAGACUGAUG	123
UAGCUUAUCAGACUGAUGUUGACUGU	104
UAGCUUAUCAGACUGAUGU	89
UAGCUUAUCAGACUGAUGUUG	62
UAGCUUAUCAGACUG	45
UAGCUUAUCAGACUGAUGUU	44
UAGCUUAUCAGACUGAUGUUGACU	21

- IsomiR levels correlated with Illumina small RNA-Seq



Bioinformatics interface

- Automated analysis of raw IzoSeq sequencing data (FastQ) to generate isomiR abundance.
- GUI developed



Future Directions

- Secure additional business/academic collaborations and funding for commercialisation
- Further optimisation → absolute quantification
- Process samples – **If you have any RNA you would be interested to know the isomiR content of then please speak to us!**

- Contact: david.simpson@qub.ac.uk for more information